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Original Article

Trichophyton verrucosum infection in livestock in the Chitral district of Pakistan

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Abstract

Introduction: *Trichophyton verrucosum* belongs to the dermatophyte fungi, closely related organisms that cause skin infections in animals and humans. *T. verrucosum* infection has been reported in livestock and people in different countries from all continents. Human cases have been reported in different areas of Pakistan, but there is little information about the animal source of the fungus.

Methodology: Dermatological specimens collected in the Chitral district of Pakistan for a study on mange in livestock were retrospectively analyzed for the presence of *T. verrucosum*. In total, 5,873 animals (1,087 cows, 2,033 goats, and 2,753 sheep) were screened for evidence of dermatological lesions during two surveys performed in the summer and winter seasons. Skin scrapings collected from animals with lesions were analyzed by direct microscopic examination after digestion in sodium hydroxide and a real-time polymerase chain reaction (PCR) targeting pathogenic *Trichophyton* species.

Results: At microscopy, samples from 18 cows (1.6%), 3 sheep (0.1%), and 4 goats (0.2%) were positive for fungal elements consistent with *T. verrucosum*. PCR confirmed the microscopy results. The prevalence was lower than that reported in other countries in intensive breeding farms. Results agree with the literature regarding factors affecting *T. verrucosum* diffusion, *i.e.*, infection was more prevalent in cattle, especially in younger animals during the winter season.

Conclusions: This study reports, for the first time, the presence of *T. verrucosum* in livestock in Pakistan. A better knowledge of the animal role in the spread of this fungus may allow the adoption of more efficient control measures and prophylaxis.

Key words: *Trichophyton verrucosum*; cattle; sheep; goat; ringworm; zoonosis.

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Introduction

Trichophyton verrucosum belongs to the dermatophyte fungi, a group of closely related organisms that have the ability to invade the stratum corneum of the epidermis and keratinized tissues derived from it, such as skin, nail, and hair of humans and animals. They produce an infection called dermatophytosis, commonly referred to as ringworm or tinea [1]. Dermatophytes are divided into anthropophilic, zoophilic, and geophilic species based on their primary habitat associations [2]. Anthropophilic species are primarily associated with humans and rarely infect animals. Zoophilic dermatophytes usually infect animals or are associated with animals but occasionally infect humans. *T. verrucosum* is included in this group and is strictly associated with cattle [3]. Other zoophilic dermatophytes of interest are *Microsporum canis*, associated with cats and dogs, and *Trichophyton mentagrophytes*, associated with rabbits, guinea pigs, and rats. Geophilic dermatophytes are primarily associated with keratinous materials spread in the environment from living animals. They have, with few exceptions (*Microsporum gypseum*), little or no pathogenic value [2,3].

Dermatophytosis is a major problem in veterinary medicine. Contagiousness among animal communities, high cost of treatment, and lack of control measures all account for its particular relevance, also in light of the public health consequences of animal ringworm.

Indeed, all animal-associated dermatophytes are transmissible to humans [3,4].

Ringworm is a common disease of cattle, with enzootic situations frequently occurring in herds worldwide. Dermatophytosis occurs less frequently in sheep and goats. As stated, *T. verrucosum* is responsible for the majority of cases [3-7].

T. verrucosum is mainly transmitted through direct contact with infected animals; therefore, high prevalence levels often occur in overcrowded stables where the fungus can spread easily among subjects confined in small areas. Furthermore, the high resistance of the dermatophyte conidia for months, or even years, in the environment leads to possible episodes of infection indirectly from contaminated fomites [3].

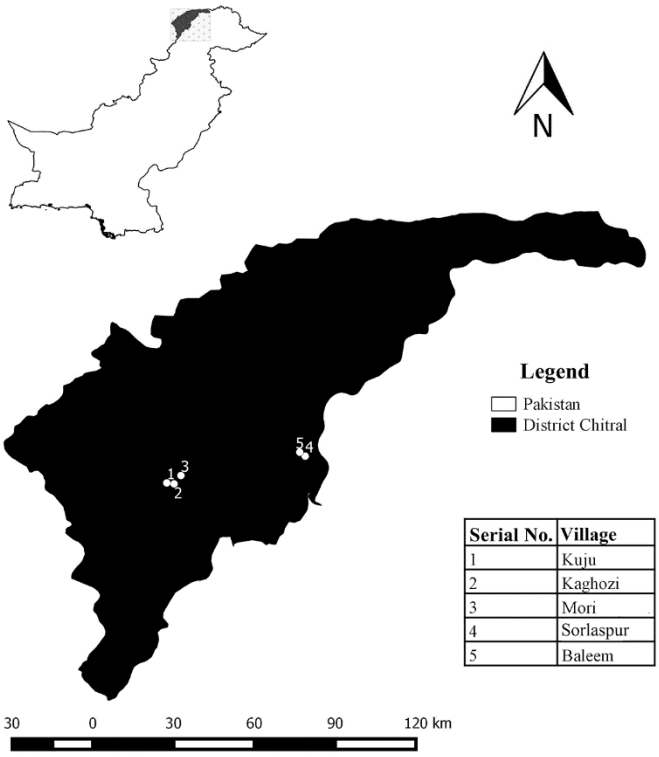
Although frequently considered as a benign self-healing infection, ringworm in cattle may be responsible for economic losses due to the negative impact on milk and meat production. Ringworm also leads to impairments in the hide and skin industries, as lesion scars are evident on leather following tawing and tanning [3,8]. Moreover, as mentioned, *T. verrucosum* is characterized by a high zoonotic potential. People at higher risk of infection are farmers and their families, and veterinarians and technicians involved in animal management. Human patients usually develop aggressive inflammatory skin lesions, which may be accompanied by constitutional symptoms such as fever and lymphadenopathy [9].

In cattle, ringworm is usually more widespread in young animals because of their lack of specific immunity against the fungus [3]. The infection is often evident, with alopecic areas covered with thin farinaceous desquamations, or with thick crusty lamellar scales difficult to pull off the skin. Lesions are mainly distributed on the head and neck, but in more severe cases, the whole body can be affected [3,10]. The possibility of asymptomatic infections has been also reported [5]. In sheep, lesions are quite similar to those described in cattle, but they preferentially involve the hairy skin, notably on the head, while not affecting the wool [3].

Diagnosis is generally made by direct microscopic examination of crusts and scales in which, after digestion with sodium hydroxide (NaOH) or potassium hydroxide (KOH), it is possible to observe the large spores (called arthroconidia) typical of this fungal species [3]. Culture, still considered to be the gold standard for the diagnosis of animal dermatophytosis [3,11], is frequently problematic regarding *T. verrucosum*, mainly due to the development of a great variety of contaminant molds in primary cultures despite the use of specific selective media. Moreover, most *T. verrucosum* isolates require thiamine, or thiamine and inositol, though some autotrophic variants that do not require an exogenous source of vitamins, have been described [3]. Recently, a number of molecular methods have also been proposed as alternative tools to detect dermatophytes, including *T. verrucosum* directly from clinical samples [12].

T. verrucosum infection can be considered to be a cosmopolitan disease as, over time, it has been reported in livestock and sometimes in people in a number of different countries from all continents [5-11,13-25]. In Pakistan, several studies have documented the role of this fungal species in human ringworm cases [26-31]. However, data are still lacking concerning the animal reservoirs of this fungus. In farm animals, greater emphasis has traditionally been given to the study of skin diseases of parasitic origin, such as mange and tick infestation [32-34]. The availability of dermatological samples collected during a recent research campaign on mange in livestock in the Chitral district of Pakistan prompted us to perform this retrospective study aimed at evaluating the presence and grade of diffusion of *T. verrucosum* in ruminants reared in this region.

Figure 1. Map of the localities in which the study was conducted.



Serial No.	Village
1	Kuju
2	Kaghozi
3	Mori
4	Sorlaspur
5	Baleem

Methodology

Study area

The study was conducted in five localities (Kaghozi, Kuju, Mori, Solaspur and Baleem) of the Chitral district. This is the northernmost and largest district in the Khyber Pakhtunkhwa (KPK) province, Pakistan. Quantum Geographical Information System (QGIS) was used to prepare a map of localities surveyed (Figure 1). The Chitral district is situated in the Hindu Kush-Pamir mountain range and shares its borders with Afghanistan (north and west), Gilgit-Baltistan (east) and Swat and Dir (south). Chitral is prominently arid with very cold winters and mild summers, and an average rainfall of 500–650 mm. The topography is characterized by high rugged mountains with narrow valleys along the sides of rivers and major tributaries. These valleys are the only places suitable for human settlements and agriculture. The main source of revenue for the rural community of Chitral is livestock rearing and subsistence farming. Most households of the community hold small herds composed of one or two cows and up to ten sheep and goats. Native mixed breeds with low productivity are generally present. During the summer season, animals, except for lactating cattle, are taken to alpine pastures where they are kept in large groups in provisory sheds made of wood and stone, built for protection against predators. In winter, each household keeps its own herd in stables close to their houses.

Sample collection

Each locality was surveyed during the summer (June–July 2012) and the winter (December 2012–January 2013). A self-developed questionnaire was used to interview collaborating farmers; it contained information on livestock species, age, gender, herd size, herd composition, and season. All animals were screened for evidence of dermatological lesions (*e.g.*, crusts, scars, alopecia, or inflammation). Skin scrapings were performed at the affected sites of symptomatic animals using a sterile scalpel blade. The blade and the material collected were preserved in 70% ethanol in 50 mL Falcon tubes.

Laboratory procedures

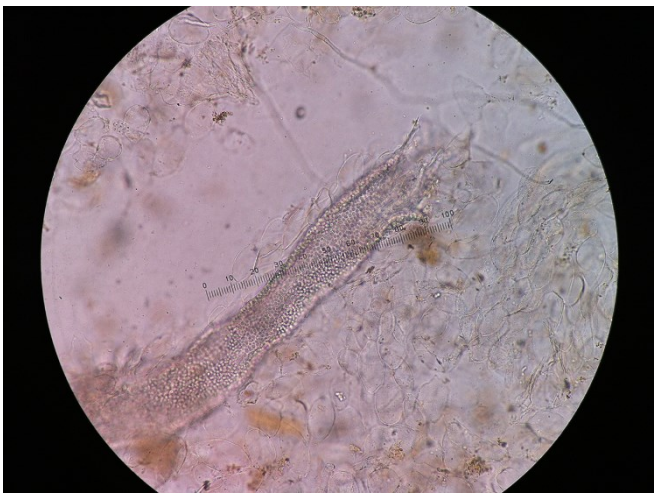
Samples were centrifuged at 3,000 rpm for 10 minutes, followed by removal of ethanol and digestion of the material in 10% NaOH for 3 hours at room temperature. After digestion, samples were centrifuged at 3,000 rpm for 10 minutes and supernatants were removed. Material present at the bottom of the tubes was transferred to slides (about 10 for each sample) and

Table 1. Rates of infection by *T. verrucosum* in animals in different localities of Chitral district.

		Summer			Winter			Total		
Locality	**Species**	**N**	**Inf**	**%**	**N**	**Inf**	**%**	**N**	**Inf**	**%**
Kuju	Sheep	257	0	0.00	296	0	0.00	553	0	0.00
	Goat	176	0	0.00	192	0	0.00	368	0	0.00
	Cattle	98	2	2.04	104	4	3.85	202	6	2.97
	Total	**531**	**2**	**0.38**	**592**	**4**	**0.68**	**1,123**	**6**	**0.53**
Kaghozi	Sheep	235	0	0.00	250	0	0.00	485	0	0.00
	Goat	201	0	0.00	179	4	2.23	380	4	1.05
	Cattle	146	1	0.68	134	3	2.24	280	4	1.43
	Total	**582**	**1**	**0.17**	**563**	**7**	**1.24**	**1,145**	**8**	**0.70**
Mori	Sheep	273	0	0.00	282	3	1.06	555	3	0.54
	Goat	217	0	0.00	187	0	0.00	404	0	0.00
	Cattle	59	0	0.00	89	2	2.25	148	2	1.35
	Total	**549**	**0**	**0.00**	**558**	**5**	**0.90**	**1,107**	**5**	**0.45**
Solaspur	Sheep	193	0	0.00	261	0	0.00	454	0	0.00
	Goat	169	0	0.00	205	0	0.00	374	0	0.00
	Cattle	139	0	0.00	176	2	1.14	315	2	0.63
	Total	**501**	**0**	**0.00**	**642**	**2**	**0.31**	**1,143**	**2**	**0.17**
Baleem	Sheep	332	0	0.00	374	0	0.00	706	0	0.00
	Goat	263	0	0.00	244	0	0.00	507	0	0.00
	Cattle	59	0	0.00	83	4	4.82	142	4	2.82
	Total	**654**	**0**	**0.00**	**701**	**4**	**0.57**	**1,355**	**4**	**0.30**
Total	**Sheep**	**1,290**	**0**	**0.00**	**1,463**	**3**	**0.21**	**2,753**	**3**	**0.11**
	Goat	**1,026**	**0**	**0.00**	**1,007**	**4**	**0.40**	**2,033**	**4**	**0.20**
	Cattle	**501**	**3**	**0.60**	**586**	**15**	**2.56**	**1,087**	**18**	**1.66**
	Total	**2,817**	**3**	**0.11**	**3,056**	**22**	**0.72**	**5,873**	**25**	**0.43**

Inf: infected.

Figure 2. Hair infected with large arthroconidia in a cow with *T. verrucosum* infection. Microscopic observation after NaOH digestion (magnification 10 X).

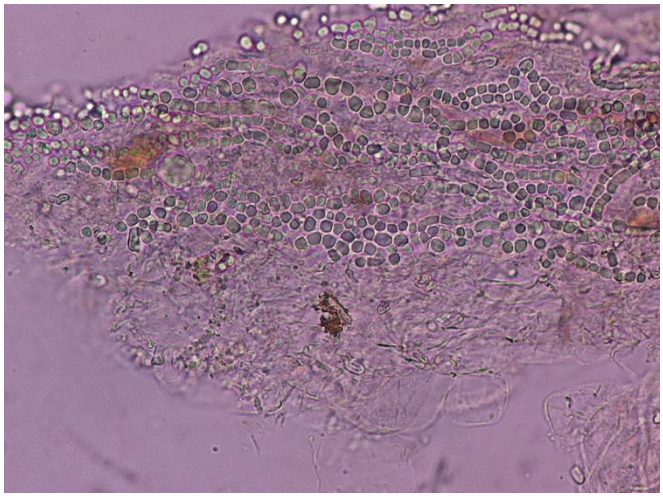


observed for the presence of fungal elements. To confirm results obtained by direct examination, a polymerase chain reaction (PCR)-based technique was employed. DNA from the clinical specimens was extracted using DNAzol reagent (Life Technologies, Monza, Italy) [35]. Samples were analyzed by real-time PCR using primers and probes developed by Arabatzis *et al.* [12] (forward primer: CTGCGGAAGGATCATTAAC; reverse primer: AAGAGATCCGTTGTTGAAAG; probe: GAGGCAACCGAGTAA). A minor groove binder (MGB) Taqman probe labeled with a VIC reporter dye at the 5'-end (Life Technologies, Monza, Italy) was used. This set of primers and probe is known to detect all pathogenic species of *Trichophyton*, including *T. verrucosum* [12]. Amplification reactions contained 10 µL DNA extract, 25 µL of 2 X Taqman Universal Master Mix (Life Technologies, Monza, Italy), 0.4 µmol L^{-1} of each primer and 0.1 µmol L^{-1} of the probe, with nuclease-free water up to a final volume of 50 µL. Cycling conditions for the PCR reaction consisted of an initial step for polymerase activation of 15 minutes at 95°C, followed by 45 amplification cycles for 30 seconds at 95°C and 1 minute at 55°C. Amplification, detection, and data analysis were performed using the ABI Prism 7500 real-time detection system (Applied Biosystems, Foster City, USA).

Statistical analysis

Risk factor analyses were performed considering animal species, locality, sex, age (< 1 year versus ≥ 1 year), herd size (< 10 versus ≥ 10 animals), and season (summer versus winter), using the Chi-square test in Epi Info 7 (Centers for Disease Control, Atlanta, USA).

Figure 3. Chains of arthroconidia of *T. verrucosum* at higher magnification (40 X). Sample obtained from cattle. Microscopic observation after NaOH digestion.

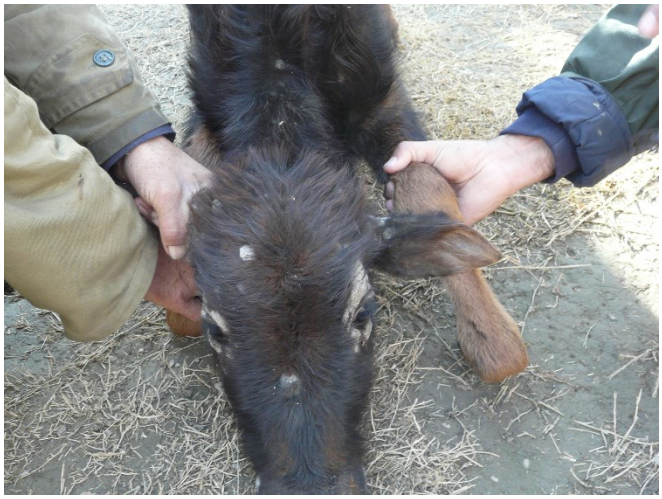


The strength of association of each factor with fungal infection was estimated through odds ratio and the corresponding 95% confidence intervals. A p value of < 0.05 was considered to be statistically significant.

Results

Overall, 25 samples from 5,873 animals screened (0.43%) resulted positive for fungal elements at microscopy (Table 1). These results were confirmed by real-time PCR. The fungal structures observed (chains/groups of large arthroconidia surrounding the hair shafts) allowed identification of *T. verrucosum* as the sole pathogenic fungus present in positive samples (Figures 2 and 3). Ringworm lesions were mostly detected on the head in the form of typical alopecic patches covered with thin desquamations or thick crusts

Figure 4. Cattle ringworm due to *T. verrucosum* with typical periocular lesions: regular alopecia and thin farinaceous scales.



(Figure 4), although more widespread lesions were occasionally observed (Figure 5).

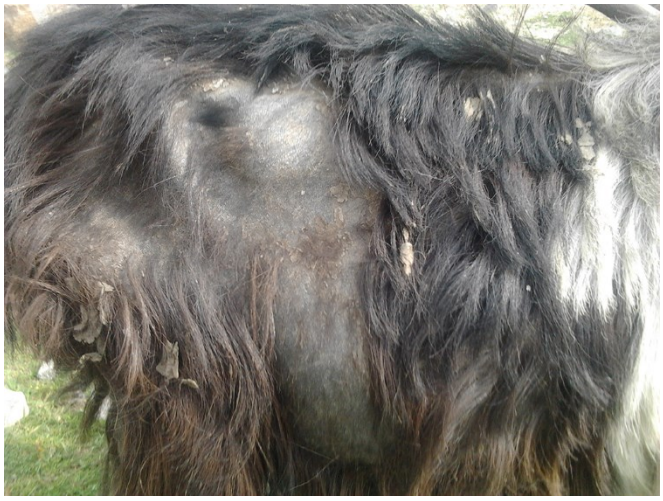
The majority of cases was found in cattle, in which the overall rate of infection (18/1,087; 1.66%) was significantly higher compared with that in sheep (3/2,753; 0.11%) ($\chi^2 = 34.29$, $p < 0.001$) and goats (4/2,033; 0.2%) ($\chi^2 = 21.52$, $p < 0.001$). The few cases involving small ruminants were all concentrated in two villages and only during the winter period (3 sheep in Mori and 4 goats in Kaghozi). Conversely, positive cattle were found in all the localities surveyed, at least in the winter period.

Due to the low prevalence of infection in sheep and goats, particularly when considering the total number of animals screened, statistical analyses were only performed on results of cattle (Table 2). Ringworm infection was significantly associated with age, with a higher prevalence in younger animals, and season, with the majority of cases found in winter. With respect to the provenance of cases, a significant difference was found only for two villages (Kuju versus Solaspur). There was no significant association with any of the other factors considered.

Discussion

This study documents, for the first time, the presence of *T. verrucosum* in domestic ruminants in Pakistan. This finding was not unexpected since this fungal pathogen has been isolated from cases of human ringworm in different localities of this country [26-31]. Our research was based on the analysis of samples originally collected for other purposes, *i.e.*, to investigate skin diseases of parasitic origin in livestock. Consequently, given that samples were stored in ethanol, it was not possible to perform mycological cultures. This obviously represents a limitation,

Figure 5. Goat ringworm due to *T. verrucosum* with extensive alopecia and scales.



although some considerations can be taken into account that decrease the importance of the lack of these results. Firstly, in the course of dermatophytosis by *T. verrucosum* in animals, diagnosis based on direct examination has a very high sensitivity [5,14]. Indeed, infected crusts and scales generally contain many fungal elements that can be easily identified, provided that effective digestion by NaOH or KOH is carried out. Secondly, we further augmented the performances of this type of test by processing a large quantity of material collected by skin scrapings from each animal. Finally, results obtained with microscopic examination were confirmed by real-time PCR, which is known to possess a sensitivity and a specificity comparable or even superior to that of culture [12]. The PCR employed has been proven to detect different species of *Trichophyton* [12], so that the exact fungal identification was based on the morphological features of fungal elements visualized at the microscopic level.

Table 2. Statistical analysis of results regarding *T. verrucosum* infection in cattle in the Chital district.

Variables		Infected	Healthy	Total	Prevalence (%)	
Locality	Kuju	6	196	202	2.97	$P < 0.05$ (Chi-square 4.41; odds ratio 4.79) only for Kuju vs Solaspur
	Kaghozi	4	276	280	1.43	
	Mori	2	146	148	1.35	
	Solaspur	2	313	315	0.63	
	Baleem	4	138	142	2.82	
Season	Summer	3	498	501	0.60	$P < 0.05$ (Chi-square 6.38; odds ratio 0.23)
	Winter	15	571	586	2.56	
Sex	Male	8	450	458	1.75	$P = 0.8$ (Chi-square 0.04; odds ratio 1.1)
	Female	10	619	629	1.59	
Age	< 1year	14	165	179	7.82	$P < 0.001$ (Chi-square 50.02; odds ratio 19.18)
	> 1year	4	904	908	0.44	
Herd size	< 10 animals	8	309	317	2.52	$P = 0.15$ (Chi-square 2.07; odds ratio 1.97)
	> 10 animals	10	760	770	1.30	

This was possible as *T. verrucosum* presents with very typical features, *i.e.*, chains of large (about 10 µm size) arthroconidia, which allows differentiation of this fungus from other dermatophyte species that potentially affect livestock [5]. The use of a molecular tool also helped to overcome the limitations of cultural examination. Indeed, it is commonly known that the poor growth of *T. verrucosum* may represent a serious problem for its isolation and identification, especially due to the rapid development of a great variety of non-pathogenic molds that contaminate hair and crusts of large animals [3,7]. In our experience, *T. verrucosum* even grows slowly in culture media supplemented with thiamine and inositol, and sometimes the growth is barely appreciable after four weeks of incubation [36].

As mentioned, only the typical pattern of hair invasion by *T. verrucosum* was detected. However, the hair microscopic examination has lower sensitivity in cases of infections by other dermatophyte species [7]. Moreover, the PCR employed does not detect *Microsporum* spp. [12]. Accordingly, we cannot definitively rule out that some animals deemed negative harbored, for example, *M. canis* or *M. gypseum*.

Our findings indicate that in Pakistan cattle are the main reservoir of *T. verrucosum*, as previously shown worldwide [3,5-7,10,13,14]. The discovery of the pathogen in all the localities visited during the survey, although with quite a low prevalence and only in the winter period, indicates that the disease is probably diffused in all the Chitral district. The low infection rate detected is likely due to the breeding system typical of this rural area, with small herds composed of one or two cows of low productivity native mixed breeds. In such a context, the fungus does not find the conditions which are known to promote its spread, such as overcrowding of animals and high humidity, that are more typically encountered in intensive breeding [5].

The analysis of literature shows that in most publications about other countries, infection rates appear considerably higher than the values found in our study. In some cases, these values attest an actual spread of the pathogen due to the factors linked to typology of animals and breeding systems, and/or to local climatic factors. It is the case, for example, of some surveys performed in intensive and semi-intensive farms in central Italy, which detected infection rates of 19% [10], 60% [5], and 88% [7]. The survey that reported the highest prevalence [7] was based on the analysis of only young animals living in crowded environments. In addition, the investigation was carried out during winter months. Therefore, the high *T. verrucosum* positivity rate was probably due to the association of risk factors present in the calf population examined. Some differences in the isolation rate may also be attributed to the fact that, in some cases, animals without any evidence of dermatological lesions were sampled as well. For example, in the aforementioned studies [5,7] *T. verrucosum* was isolated, respectively, from 80.4% and 15% of asymptomatic animals. Thus, it should be noted that, in the present study, we could not evaluate the contribution of asymptomatic infections to the overall prevalence. This issue would be worth investigating further in a future study, not only to evaluate the real prevalence of infection, but also because asymptomatically infected subjects may play an important role in the spread of the disease to other animals and humans.

A high infection rate (31%) was also found in a study in Jordan that took into account 10 large dairy farms, which included each 200–400 animals each [37]. In this case, however, it must be noted that the prevalence value was overestimated; indeed, in the table reporting the list of fungi isolated from calves with ringworm, a considerable percentage is represented by molds, such as *Aspergillus* spp., *Alternaria* spp., *Penicillium* spp., *etc*., which should have been considered merely as skin contaminants without any pathogenic role.

Infection rates higher than those found in the present study were also reported in other countries: 85% in Nigeria [6], 20% in China [13], and 25% in Spain [11]. However, a deeper analysis reveals that these studies are not comparable with ours and that the high positivity rates reported do not represent a realistic indication of the importance of *T. verrucosum* infection in these countries. Indeed, the study performed in Nigeria [6] described an outbreak in a small farm, with 12 infected animals out of a total of 14. Likewise, the infection rate reported in the study in China [13] only reflects the prevalence within an outbreak in a single farm, but on a larger scale, with 200 animals infected out of a total of 1,000. Finally, the Spanish study [11] presented a completely different experimental approach, as it concerned the retrospective description of the activity of a mycology diagnostic service. Moreover, the rate of infection reported (25%) appears far from being representative of the actual epidemiological situation in Spain. Indeed, this datum corresponds to one positive sample out a total of four examined over a 10-year period.

To finish this comparison with existing literature, it is worth noting that one study performed in Iran [14], which is comparable with ours regarding experimental approach and geographical/social context, reported a

much lower prevalence (around 5%), closer to the value obtained in our study.

In the present study, sheep and goats were also occasionally affected, with significantly lower frequency and only in the winter period. Moreover, all cases were concentrated in two villages (Table 1). These results contribute to reinforce the widely accepted opinion that ringworm is less frequent in small ruminants [3,4] perhaps due to a stronger inherited immune response against the fungus compared with that of cattle, or to other factors linked to the breeding systems. It is, however, important to note that some publications have documented, in the sheep in other countries (such as the United Kingdom, the United States, and Morocco), an increasing prevalence of the disease and the existence of extensive outbreaks [19,38-40]. Moreover, it cannot be excluded that dermatophytosis in sheep and goats is an under-diagnosed disease and/or that many infection episodes are simply not reported in the official literature.

The analysis of our results reveals that, in this district of Pakistan, the dynamics of transmission of *T. verrucosum* in cattle do not appear to differ from those described elsewhere. For example, the infection was significantly associated with younger animals, probably related to the absence of specific immunity, which generally develops in older animals following repeated exposure to the fungus [3,5]. Moreover, a significantly higher number of cases was found in the winter season. This predominance is a typical feature of ringworm in cattle, in which skin lesions are more frequently present in winter when animals are confined to stables, as well as because of the higher humidity, and lesions tend to disappear spontaneously after turn out [3,4]. Also, the lack of association of the fungal infection with sex confirms what was previously reported [3,5,14].

Due to the retrospective nature of this research, it was impossible to quantify the importance of *T. verrucosum* infection in humans. While further studies are necessary to clarify this issue, different considerations lead to the assumption that human involvement plays a role in the area sampled. Firstly, a previous study performed in the villages of the same district revealed the presence of some cases of human ringworm, although the exact identification of the fungal species involved was not obtained [41]. Secondly, the social context in which we operated, namely a rural community where there were occasions for contact with potentially infected livestock, resembles the situation described in a previous study in another locality of Pakistan (Karachi), where a high proportion (about 25%) of human ringworm episodes were due to *T. verrucosum* [31]. Finally, during our visits to the villages, we noted that there was little awareness of zoonotic risk among people in contact with animals. This is exemplified by Figure 4, which shows the habitude of handling animals, even those with evidence of dermatological lesions, without adopting any protection.

Conclusions

This study has enabled to obtain data on the diffusion of *T. verrucosum* infection in livestock in Pakistan. This contribution may represent a good starting point for a better comprehension of transmission dynamics in the case of human infections due to this zoophilic dermatophyte. Improved knowledge may ultimately facilitate the adoption of more efficient control measures and prophylaxis.

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